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(54) Title: SCORPION PEPTIDE WITH IMMUNOSUPPRESSANT ACTIVITY			
(57) Abstract  A thirty-nine amino acid peptide, Margatoxin (MgTX), is purified to homogeneity from venom of the scorpion <i>Centruroides margaritatus</i> . The gene encoding MgTX is constructed and this gene is expressed in <i>E. coli</i> , to produce recombinant MgTX. MgTX is a potent and selective inhibitor of a voltage-dependent K <sup>+</sup> channel present in human lymphocytes. MgTX exhibits immunosuppressant activity with human T-lymphocytes, and is useful as an immunosuppressant, in modeling nonpeptidyl K <sup>+</sup> channel blockers, and in establishing biochemical assays based on ligand binding or other protocols with which to screen for other novel modulators of voltage dependent K <sup>+</sup> channels in lymphocytes and other tissues including the brain. As an immunosuppressant, MgTX is useful in the treatment of autoimmune diseases, the prevention of rejection of foreign organ transplants and/or related afflictions, diseases and illnesses.			

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TITLE OF THE INVENTION

## SCORPION PEPTIDE WITH IMMUNOSUPPRESSANT ACTIVITY

SUMMARY OF THE INVENTION

5 A thirty-nine amino acid peptide, Margatoxin (MgTX), is purified to homogeneity from venom of the scorpion Centruroides margaritatus. The gene encoding MgTX is constructed, and this gene is expressed in E. coli to produce recombinant MgTX. MgTX is a potent and selective inhibitor of a voltage-dependent K<sup>+</sup> channel present in 10 human lymphocytes. MgTX exhibits immunosuppressant activity with human T-lymphocytes, and is useful as an immunosuppressant, in modeling nonpeptidyl K<sup>+</sup> channel blockers, and in establishing biochemical assays based on ligand binding or other protocols with which to screen for other novel modulators of voltage dependent K<sup>+</sup> 15 channels in lymphocytes and other tissues including the brain. As an immuno-suppressant, MgTX is useful in the treatment of autoimmune diseases, the prevention of rejection of foreign organ transplants and/or related afflictions, diseases and illnesses.

BACKGROUND OF THE INVENTION

20 Immunoregulatory abnormalities have been shown to exist in a wide variety of "autoimmune" and chronic inflammatory diseases, including systemic lupus erythematosus, chronic rheumatoid arthritis, type I and II diabetes mellitus, inflammatory bowel disease, biliary 25 cirrhosis, uveitis, multiple sclerosis and other disorders such as Crohn's disease, ulcerative colitis, bullous pemphigoid, sarcoidosis, psoriasis, ichthyosis, and Graves ophthalmopathy. Although the underlying pathogenesis of each of these conditions may be quite different, they have in common the appearance of a variety of auto antibodies and self- 30 reactive lymphocytes. Such self-reactivity may be due, in part, to a loss of the homeostatic controls under which the normal immune system operates.

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Similarly, following a bone-marrow or an organ transplantation, the host lymphocytes recognize the foreign tissue antigens and begin to produce antibodies which lead to graft rejection.

5 One end result of an autoimmune or a rejection process is tissue destruction caused by inflammatory cells and the mediators they release. Anti-inflammatory agents such as NSAID's and corticosteroids act principally by blocking the effect or secretion of these mediators but do nothing to modify the immunologic basis of the disease. On the  
10 other hand, cytotoxic agents, such as cyclophosphamide, act in such a nonspecific fashion that both the normal and autoimmune responses are shut off. Indeed, patients treated with such nonspecific immuno-suppressive agents are as likely to succumb from infection as they are from their autoimmune disease.

15 Cyclosporin A, which was approved by the US FDA in 1983, is currently the leading drug used to prevent rejection of transplanted organs. The drug acts by inhibiting the body's immune system from mobilizing its vast arsenal of natural protecting agents to reject the transplant's foreign protein. Though cyclosporin A is effective in fighting transplant rejection, it is nephrotoxic and is known  
20 to cause several undesirable side effects including kidney failure, abnormal liver function and gastrointestinal discomfort.

Newer, safer drugs exhibiting fewer side effects are constantly being searched for in the field. The present invention provides for an immunosuppressant peptide that is a selective inhibitor  
25 of a voltage dependent potassium channel,  $K_v1.3$ , that is found on human T-lymphocytes.

Potassium channels modulate a number of cellular events such as muscle contraction, neuro-endocrine secretion, frequency and duration of action potentials, electrolyte homeostasis, and resting  
30 membrane potential. These channels comprise a family of proteins that have been classified according to their biophysical and pharmacological characteristics. Inhibition of  $K^+$  channels, in their role as modulators of the plasma membrane potential in human T-lymphocytes, has been postulated to play a role in eliciting immunosuppressive responses. In

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regulating membrane potential, K<sup>+</sup> channels play a role in the regulation of intracellular Ca<sup>++</sup> homeostasis, which has been found to be important in T-cell activation. The biochemical characterization of K<sup>+</sup> channels is underdeveloped, due to the paucity of selective high affinity probes.

Functional voltage-gated K<sup>+</sup> channels can exist as multimeric structures formed by the association of either identical or dissimilar subunits. This phenomena is thought to account for the wide diversity of K<sup>+</sup> channels found in different tissues. Despite the rapid advances in the molecular biology of K<sup>+</sup> channels, subunit compositions of native K<sup>+</sup> channels and the physiologic role that particular channels play are, in most cases, still unclear. To address these problems, potent, selective probes for channels of interest must be identified.

The K<sub>v</sub>1.3 channel is a voltage-gated potassium channel that is found in neurons, blood cells, osteoclasts and T-lymphocytes. The Chandy and Cahalan laboratories proposed a hypothesis that blocking the K<sub>v</sub>1.3 channel would illicit an immunosuppressant response. (Chandy *et al.*, *J. Exp. Med.* 160, 369, 1984; Decoursey *et al.*, *Nature*, 307, 465, 1984). However, the K<sup>+</sup> channel blockers employed in their studies were non-selective. Until the present invention, no specific inhibitor of the K<sub>v</sub>1.3 channel existed to test this hypothesis. Although a laboratory (Price *et al.*, *Proc. Natl. Acad. Sci. USA*, 86, 10171, 1989) showed that charybdotoxin would block K<sub>v</sub>1.3 in human T cells, charybdotoxin was subsequently shown to inhibit four different K<sup>+</sup> channels (K<sub>v</sub>1.3 and three distinct small conductance Ca<sup>2+</sup> activated K<sup>+</sup> channels) in human T lymphocytes, limiting the use of this toxin as a probe for the physiological role of K<sub>v</sub>1.3 (Leonard *et al.*, *Proc. Natl. Acad. Sci. USA*, 89, 10094, 1992). Since MgTX is a selective K<sub>v</sub>1.3 inhibitor, it is useful to show that blocking of K<sub>v</sub>1.3 will inhibit T cell activation (Lin *et al.*, *J. Exp. Med.* 177, 637, 1993).

Scorpion venoms have been recognized as a source of peptidyl inhibitors of various types of K<sup>+</sup> channels. Some of these peptides have been purified to homogeneity and their properties characterized. A preliminary report describing the presence of a

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specific peptidyl voltage-gated potassium channel inhibitor in *C. margaritatus* venom that is homologous with NxTX has been made in abstract form (Novick, et al. (1991) *Biophys J* 59, 78). The most extensively studied of these toxins is charybdotoxin (ChTX). See U.S. Patent No. 4,906,867. ChTX is a thirty-seven amino acid peptide isolated from venom of the old world scorpion Leiurus quinquestriatus var. hebraeus. Originally described as an inhibitor of the high-conductance,  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  (Maxi-K) channel present in muscle and neuro-endocrine cells, ChTX was later found also to inhibit a number of different medium- and small-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels, as well as a voltage-dependent  $\text{K}^{+}$  channel ( $\text{K}_v1.3$ ). In each case, channel inhibition occurs with similar potency, in the low nanomolar range. Therefore, caution has to be exercised when using ChTX to study the physiological role of a given channel in a tissue of interest. A related toxin, iberiotoxin (IbTX), shares 68% sequence homology with ChTX and selectively blocks the Maxi-K channel. Other peptidyl inhibitors, such as limbatustoxin (LbTX) and kaliotoxin (KTX), have also been shown to possess greater selectivity for the Maxi-K channel. Other peptidyl toxins homologous to ChTX have been identified (e.g., noxiustoxin). Noxiustoxin is a high-affinity blocker of  $\text{K}_v1.3$ , but because it also inhibits the delayed rectifier  $\text{K}^{+}$  channel of squid axon and the Maxi-K channel from skeletal muscle, and is not selective for  $\text{K}_v1.3$ , it is not a satisfactory probe of  $\text{K}_v1.3$  channels.

The peptide of the present invention, MgTX, represents a unique tool with which to probe the function of  $\text{K}_v1.3$ . This channel has been identified as the major voltage-dependent  $\text{K}^{+}$  conductance in peripheral human T-lymphocytes. Since human T-lymphocytes contain, in addition to  $\text{K}_v1.3$ , several distinct small-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels that are also blocked by ChTX, ChTX is inadequate for assigning the role of any specific channel in the control of T cell proliferation. MgTX has recently been demonstrated to depolarize human T cells (Leonard et al., *Proc. Natl. Acad. Sci. U. S. A.* 89, 10094, 1992) and to prevent activation and proliferation of these cells

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mediated by  $\text{Ca}^{2+}$ -dependent pathways (Lin *et al.*, *J. Exp. Med.*, **177**, 637, 1993).

Venom of the new world scorpion Centruroides  
5 margaritatus was determined to contain an activity selectively directed  
against voltage-dependent  $\text{K}^{+}$  channels: it inhibited binding of  
[125I]ChTX to  $\text{K}_{\text{v}}1.3$  channels in rat brain synaptosomal membranes,  
but not to Maxi-K channels in smooth muscle sarcolemma. This  
invention comprises the purification, from this venom, of margatoxin  
10 (MgTX), its primary sequence and characterization as a  $\text{K}_{\text{v}}1.3$   
inhibitor and the expression of recombinant MgTX in E. coli. MgTX  
is structurally related to other known  $\text{K}^{+}$  channel blocking peptides, but  
is distinguished by its potent and selective blockade of  $\text{K}_{\text{v}}1.3$ . Given  
these properties, MgTX represents a useful tool for studying the  
15 physiologic role of  $\text{K}_{\text{v}}1.3$ . This invention also relates to the  
construction of a gene encoding MgTX and the expression of this gene  
in E. coli to produce recombinant MgTX.

#### DESCRIPTION OF THE DRAWINGS

20 Figure 1 depicts the plasmid map of the synthetic MgTX  
gene for expression in E. coli, the locations of the synthetic MgTX  
gene, Factor Xa cleavage site, and the T7 gene9 fusion protein. Amp<sup>r</sup>  
represents ampicillin resistance; Ori represents the origin.

#### DETAILED DESCRIPTION OF THE INVENTION

25 The invention relates to Margatoxin (MgTX), a novel 39  
amino acid peptide containing six cysteine residues and seven positively  
charged residues, derived from venom of the scorpion Centruroides  
margaritatus : (SEQ ID NO:1)

30

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Thr-Ile-Ile-Asn-Val-Lys-Cys-Thr-Ser-Pro-Lys-Gln-Cys-Leu-Pro-Pro-  
                                   5                                  10                                  15

Cys-Lys-Ala-Gln-Phe-Gly-Gln-Ser-Ala-Gly-Ala-Lys-Cys-Met-Asn-Gly-  
                                   20                                  25                                  30

Lys-Cys-Lys-Cys-Tyr-Pro-His  
                                   35

10 This invention relates to the purification of MgTX from C. margaritatus venom so that the peptide is substantially free from other peptide contaminants as judged by reversed phase HPLC, SDS-PAGE analysis, amino acid compositional analysis, N-terminal degradation sequence analysis, and electrospray mass spectroscopy. MgTX is useful as a selective probe for Kv1.3, and may be employed in a screen for  
 15 other Kv1.3 inhibitors exhibiting immunosuppressant activity. Additionally, MgTX is useful in modeling studies to design nonpetidyl Kv1.3 blockers. MgTX is also useful for its immunosuppressive activity. MgTX inhibits interleukin 2 production in human T-lymphocytes (Lin *et al.*, *J. Exp. Med.* **177**, 637, 1993).

20 This invention also relates to the construction of a gene encoding MgTX (SEQ ID NO:2) and the expression of this gene in E. coli to produce recombinant MgTX. The DNA sequence encoding MgTX is shown below: (SEQ ID NO:2)

25 5' ACCATCATCA ACGTTAAATG CACCTCTCCG AAACAGTGCC  
                   TGCCGCGGTG CAAAGCTCAG TTCGGTCAGT CTGCTGGTG  
                   TAAATGCATG AACGGTAAAT GCAAATGCTA CCCGCACTGA 3'

30 It is also well-known that there is a substantial amount of redundancy in the various codons which code for specific amino acids. Therefore, this invention is also directed to those DNA sequences which contain alternative codons which code the identical amino acid. For purposes of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variation. Also included within



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the scope of this invention are variations in the DNA sequence which do not alter the ultimate physical properties of the expressed protein.

This invention also concerns systems for expressing MgTX. This system includes expression vectors which incorporate a base sequence encoding MgTX. This expression vector is then used to transfect a suitable expression host, preferably *E. coli*, which translates the genetic information, synthesizes the protein or partial protein and enables expression of the cloned MgTX. Figure 1 depicts the plasmid map of the synthetic MgTX gene for expression in *E. coli*, the locations of the synthetic MgTX gene, Factor Xa cleavage site, and the T7 gene9 fusion protein.

Because the amounts of MgTX that can be obtained from scorpion venom are small, the ability to produce large amounts of peptide by recombinant techniques allows its application in assays to develop other potassium channel inhibitors. Radiolabeling of MgTX with  $^{125}\text{I}$  Na, or derivitization with [ $^{125}\text{I}$ ] Bolton-Hunter reagent or with a biotin conjugate, allows the development of a binding assay to screen for either competitive or allosteric modulators of peptide binding that might possess  $\text{K}_v1.3$  channel inhibitory activity. MgTX can also be used as a positive control in any functional assay to discover inhibitors of  $\text{K}_v1.3$ .

MgTX peptide is isolated from crude venom of the scorpion *C. margaritatus*. Preferably, lyophilized venom is employed. The lyophilized venom is suspended in a basic, low ionic strength buffer, pH 8 to 10, preferably 20 mM sodium borate buffer at pH 9.0 at a concentration of 5 mg/mL. The venom is agitated, preferably by vortex, and clarified, preferably by centrifugation at between 20,000 xg to 30,000 xg for about 10 to 20 minutes, most preferably at 27,000 xg for 15 minutes. The supernatant is removed and preferably is filtered through 0.22  $\mu\text{m}$  low protein binding filters made of hydrophilic DURAPORE (PVDF) membrane to remove particulates that would clog ion exchange systems, such as FPLC systems, preferably through a MILLEX-GV 0.22  $\mu\text{m}$  filter, and loaded onto an ion exchange system, preferably an FPLC system in the buffer originally employed to

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dissolve the venom. Most preferably, the supernatant is loaded onto a MonoS FPLC column (HR5/5; PHARMACIA), equilibrated with 20 mM sodium borate, pH 9.0. Absorbance is measured at 280 nm. After the absorbance of the eluate returns to baseline, bound material is eluted  
5 from the resin by employing a salt gradient, preferably with a linear gradient of NaCl (0.75 M/hr) at a flow rate of 0.5 mL/min. Fractions are collected and separated and assayed for their activity against K<sup>+</sup> channels. Preferably, the ability of the fractions to inhibit [<sup>125</sup>I]ChTX binding to either rat brain synaptic plasma membranes (K<sub>v</sub>1.3 channels;  
10 Vazquez *et al.*, *J. Biol. Chem.* **265**, 15564, 1990) or bovine aortic smooth muscle sarcolemmal membranes (Maxi-K channels; Vazquez *et al.*, *J. Biol. Chem.* **264**, 20902, 1989) is measured. Fractions that block [<sup>125</sup>I]ChTX binding to brain membranes and not to smooth  
15 muscle sarcolemmal membranes and that display the highest absorbance at 280 nm are loaded onto a reversed-phase HPLC system and eluted along a linear gradient. Preferably, the material is loaded onto a 300 Å pore size C18 reversed phase HPLC column (0.46 x 25 cm) equilibrated with 10 mM trifluoroacetic acid (TFA) and eluted with a linear gradient of isopropyl alcohol/acetonitrile (2:1; 0-15%, 33 minutes) at a flow rate  
20 of 0.5 mL/min. Fractions are collected, lyophilized and reconstituted in a buffered aqueous medium, preferably 100 mM NaCl, 20 mM Tris-HCl, pH 7.4. Material exhibiting inhibitory binding activity against [<sup>125</sup>I]ChTX binding to rat brain synaptic plasma membrane vesicles is identified.

25 To determine the molecular weight of the peptide, samples are denatured in the presence of SDS, applied to gels, and the active material is analyzed using silver staining to reveal the active component. Preferably, the material is applied to precast 16% Tricine gels (NOVEX).

30 The MgTX gene may be constructed by a variety of methods including, but not limited to, cloning of the gene from scorpion DNA or RNA extracts, using the DNA sequence disclosed herein and standard recombinant DNA techniques (See Maniatis Cold Spring Harbor). An alternative is to synthesize overlapping oligonucleotides,

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followed by annealing and extension of these oligonucleotides to produce a double-stranded DNA molecule encoding the peptide sequence of MgTX, together with flanking sequence necessary to accomplish insertion of the MgTX synthetic gene into a suitable expression plasmid. Preferably, the gene is constructed beginning with two synthetic oligonucleotides of the sequences below: (SEQ ID NO: 3)

5' GTCGACGGAT CCATCGAAGG TCGTACCATC ATCAACGTTA  
AATGCACCTC TCCGAAACAG TGCCTGCCGC CGTGCAAAGC TCAG 3'  
(SEQ ID NO: 4)  
5' GGCACGTTTC GAGTCAAGCC AGTCAGACGA CCACGATTTA  
CGTACTTGCC ATTTACGTTT ACGATGGGCG TGACTATCCT TCGAA3'

These oligonucleotides may be synthesized using a commercial DNA synthesizer such as the ABI model 391 DNA Synthesizer (Applied Biosystems). The two synthetic oligonucleotides are shown above in (SEQ ID NO: 3) and (SEQ ID NO: 4), except for the 5 dCTP bases that were added to the 5' end of each to facilitate cutting with restriction enzymes.

Codon usage may be optimized for high level expression in an expression system. Suitable expression systems include: yeast, eukaryotic viral expression systems such as Baculovirus or Vaccinia virus and E. coli. The preferred expression system is E. coli BL21(DE3). The oligonucleotides are purified by affinity chromatography, preferably using NENSORB columns (DUPONT), annealed, filled in with dNTPs using SEQUENASE (U.S. BIOCHEMICAL), and digested with restriction enzymes using standard techniques. The resulting fragment is gel-purified, preferably using NUSIEVE (FMC BIOPRODUCTS) and ligated into a suitable plasmid such as pCSP105, a variant of pSR9, using standard techniques as described in Ausubel et al., eds. (1989) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York. The resulting construct,

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encoding a fusion protein of T7 gene 9 and MgTX separated by a factor X<sub>a</sub> cleavage site may be verified using dideoxy sequencing.

The expression plasmid is used to transform E. coli BL21(DE3) which are subsequently induced to produce the MgTX fusion protein by treatment with IPTG while the E. coli are in log phase growth. The cells are then harvested, washed, broken by sonication and the MgTX fusion protein can be verified by SDS-PAGE. The recombinant MgTX is cleaved from its fusion protein using Factor X<sub>a</sub> protease. Recombinant MgTX may be separated and purified according to the procedures used for isolating MgTX from crude venom.

The coding strand of the DNA from the MgTX plasmid including SaII, Factor X<sub>a</sub>, MgTX, and Hind III is shown below: (SEQ ID NO: 5).

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15  5'  GTCGACGGAT CCATCGAAGG TCGTACCATC ATCAACGTTA
      AATGCACCTC TCCGAAACAG TGCCTGCCGC CGTGCAAAGC
      TCAGTTCGGT CAGTCTGCTG GTGCTAAATG CATGAACGGT
20  AAATGCAAAT GCTACCCGCA CTGATAGGAA GCTT 3'

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The present invention is also directed to a method for suppressing the immune system in a subject in need of such treatment comprising the administration to a subject in need of such treatment of a nontoxic immunosuppressant amount of Margatoxin. Specifically, the method of this invention is useful in treating and preventing the resistance to transplantation or transplantation rejection of organs or tissues (such as heart, kidney, liver, lung, bone marrow, cornea, pancreas, intestinum tenue, limb, muscle, nervus, medulla ossium, duodenum, small-bowel, medulla ossium, skin, pancreatic islet-cell, etc. including xeno transplantation), graft-versus-host diseases by medulla ossium transplantation, autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, nephrotic syndrome lupus, Hashimoto's thyroiditis, multiple sclerosis, myasthenia gravis, type I diabetes mellitus, type II adult onset diabetes, uveitis, nephrotic

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syndrome, steroid-dependent and steroid-resistant nephrosis, Palmo-planter pustulosis, allergic encephalomyelitis, glomerulonephritis, etc., and infectious diseases caused by pathogenic microorganisms.

5           Margatoxin is also useful for treating inflammatory, proliferative and hyperproliferative skin diseases and cutaneous manifestations of immunologically-mediated illnesses such as: psoriasis, psoriatic arthritis, atopic dermatitis, contact dermatitis and further eczematous dermatitises, seborrhoeic dermatitis, Lichen planus, Pemphigus, bullous Pemphigoid, Epidermolysis bullosa, urticaria, 10           angioedemas, vasculitides, erythemas, cutaneous eosinophilias, acne, Alopecia areata, eosinophilic fasciitis, and atherosclerosis.

          Margatoxin is further useful in the treatment of respiratory diseases, for example: sarcoidosis, fibroid lung, idiopathic interstitial pneumonia, and reversible obstructive airways disease, including 15           conditions such as asthma, including bronchial asthma, allergic asthma, intrinsic asthma, extrinsic asthma and dust asthma, particularly chronic or inveterate asthma (for example late asthma and airway hyper-reponsiveness), bronchitis and the like. MgTX may also be useful for treating hepatic injury associated with ischemia.

20           MgTX may also be indicated in certain eye diseases such as keratoconjunctivitis, vernal conjunctivitis, uveitis associated with Behcet's disease, keratitis, herpetic keratitis, conical cornea, dystorpha epithelialis corneae, corneal leukoma, ocular pemphigus, Mooren's ulcer, Scleritis, Graves' ophthalmopathy, severe intraocular 25           inflammation, and the like. MgTX is also useful for treating multidrug resistance of tumor cells, (i.e. enhancing the activity and/or sensitivity of chemotherapeutic agents), preventing or treating inflammation of mucosa or blood vessels (such as leukotriene B4-mediated diseases, gastric ulcers, vascular damage caused by ischemic diseases and thrombosis, ischemic bowel disease, inflammatory bowel disease (e.g., 30           Crohn's disease and ulcerative colitis), necrotizing enterocolitis), or intestinal lesions associated with thermal burns, cytomegalovirus infection, particularly HCMV infection.

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Further, MgTX is also useful for treating or preventing renal diseases including interstitial nephritis, Goodpasture's syndrome, hemolytic-uremic syndrome and diabetic nephropathy; nervous diseases selected from multiple myositis, Guillain-Barre syndrome, Meniere's  
5 disease and radiculopathy; endocrine diseases including hyperthyroidism and Basedow's disease; hematic diseases including pure red cell aplasia, aplastic anemia, hypoplastic anemia, idiopathic thrombocytopenic purpura, autoimmune hemolytic anemia, agranulocytosis and anerythroplasia; bone diseases including osteoporosis; respiratory  
10 diseases including sarcoidosis, fibroid lung and idiopathic interstitial pneumonia; skin diseases including dermatomyositis, leukoderma vulgaris, ichthyosis vulgaris, photoallergic sensitivity and cutaneous T cell lymphoma; circulatory diseases including arteriosclerosis, aortitis syndrome, polyarteritis nodosa and myocardosis; collagen including  
15 scleroderma, Wegener's granuloma and Sjogren's syndrome; adiposis; eosinophilic fasciitis; periodontal disease; nephrotic syndrome; hemolytic-uremic syndrome; and muscular dystrophy. Further still, MgTX may be used in the treatment of diseases including intestinal inflammations/allergies such as Coeliac disease, proctitis, eosinophilic  
20 gastroenteritis, mastocytosis, Crohn's disease and ulcerative colitis; and food-related allergic diseases which have symptomatic manifestation remote from the gastrointestinal tract, for example migraine, rhinitis and eczema.

MgTX may also be useful for the treatment and prevention  
25 of hepatic diseases such as immunogenic diseases (e.g. chronic autoimmune liver diseases including autoimmune hepatitis, primary biliary cirrhosis and sclerosing cholangitis), partial liver resection, acute liver necrosis (e.g. necrosis caused by toxins, viral hepatitis, shock or anoxia), B-virus hepatitis, non-A/non-B hepatitis and cirrhosis.

30 The Margatoxin peptide may be administered intranasally, intravenously, subcutaneously or intramuscularly, preferably intravenously. Administration may occur one to four times daily or less frequently at therapeutically effective doses. Margatoxin may also be administered on an intermittent basis when therapeutically advisable.

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Dosages may be varied, depending on the age, and other conditions of patients, but daily dosage for adults is within a range of from about 0.001 mg to 10 mg, preferably 0.1 mg to 1.0 mg which may be given in a single dose or two or more divided dosages. Higher dosages may be favorably employed as required.

Margatoxin, either isolated from the venom of Centruroides margaritatus or produced by recombinant DNA technology or other means, is formulated as an injectable formulation, preferably an intravenous injection. The formulation may be formulated as either a suspension or a solution. A suspension may have the advantage of providing more gradual release of the active agent. The suspensions or solutions of the formulation of the present invention contain 0.01% to 10% Margatoxin, preferably 0.1 to 1%, in a pharmaceutically acceptable carrier.

When Margatoxin is administered as an injectable suspension or solution, the suspension or solution comprises Margatoxin and a suitable pharmaceutical carrier. Injectable suspensions and solutions may be formulated according to known art, using suitable non-toxic, parenterally acceptable diluents or solvents, such as 1,2-propanediol, water, Ringer's solution, dextrose solution or isotonic sodium chloride solution. These injectable suspensions or solutions may further contain excipients suitable for manufacture of aqueous suspensions and solutions. Such excipients may be:

- (1) suspending agents such as sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia;
- (2) dispersing or wetting agents which may be:
  - (a) a naturally occurring phosphatide such as lecithin,
  - (b) a condensation product of any alkylene oxide with a fatty acid, for example, polyoxyethylene stearate,
  - (c) a condensation product of an ethylene oxide with a long chain aliphatic alcohol, for example, heptadeca-ethyleneoxycetanol,

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- (d) a condensation product of ethylene oxide with a partial ester derived from a fatty acid and hexitol such as polyoxyethylene sorbital monooleate, or
- (e) a condensation product of ethylene oxide with a partial ester derived from a fatty acid and hexitol anhydride, for example, polyoxyethylene sorbital monooleate.

5

Such suspensions and solutions may further contain microcrystalline cellulose for imparting bulk and methylcellulose as a viscosity enhancer.

10

The aqueous suspensions and solutions may also contain one or more preservatives, for example, ethyl or n-propyl p-hydroxybenzoate, and antioxidants and the like may be incorporated as required.

15

In addition, the aqueous suspension or solution may, if necessary, be buffered to a physiologically appropriate pH by the addition of a suitable buffer such as sodium acetate, sodium lactate, sodium benzoate or Tris.

20

Dispersible powders and granules are suitable for the preparation of an aqueous suspension. They provide the active ingredient in a mixture with a dispersing or wetting agent, a suspending agent and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those already mentioned above. Additional excipients may also be present.

25

An oily suspension may be formulated by suspending the active ingredient in a vegetable oil, for example: arachis oil, olive oil, sesame oil, or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions may contain a thickening agent, for example: beeswax, hard paraffin or cetyl alcohol. These compositions may be prepared by the addition of an antioxidant such as ascorbic acid.

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The formulation of the present invention may be used in conjunction with modern auto-injection devices which increase the convenience and decrease the discomfort of subcutaneous injections. The use of the formulation of the present invention with such



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autoinjection devices may increase patient compliance by improving ease of administration.

5       The following examples are given for the purpose of illustrating the present invention and shall not be construed as being limitations on the scope or spirit of the invention.

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EXAMPLE 1Purification of Margatoxin Peptide (MgTX)

5           Crude lyophilized venom of the scorpion C. margaritatus  
obtained from MILLER INTERNATIONAL VENOMS, P.O. Box  
1644, Hollywood, Florida 33022, was suspended in 20 mM Na-borate,  
pH 9.0, at a concentration of 5 mg/mL. After agitating the venom by  
vortex, the suspension was clarified by centrifugation at 27,000xg for  
15 min. The supernatant was removed, filtered through a MILLEX-GV  
10 filter (0.2 µm pore size; MILLIPORE) and loaded onto a MonoS FPLC  
column (HR5/5; PHARMACIA), equilibrated with 20 mM sodium  
borate, pH 9.0. After the absorbance of the eluate had returned to  
baseline, bound material was eluted from the resin with a linear  
15 gradient of NaCl (0.75 M/hr) at a flow rate of 0.5 mL/min. Fractions  
were separated and assayed for their ability to inhibit [125I]ChTX  
binding to either rat brain synaptic plasma membranes or bovine aortic  
smooth muscle sarcolemmal membranes. [125I]ChTX was obtained  
from New England Nuclear Corporation. Fractions that blocked  
[125I]ChTX binding only to brain membranes and that displayed the  
20 highest absorbance at 280 nm were considered for further  
characterization. Fractions eluting at 210-340 mM NaCl displayed  
inhibitory activity against [125I]ChTX binding to Kv1.3 in rat brain  
membranes. These fractions were loaded onto a 300 Å pore size C18  
25 reversed phase HPLC column (0.46 x 25 cm) that had been equilibrated  
with 10 mM trifluoroacetic acid (TFA). The column was eluted with a  
linear gradient of isopropyl alcohol/acetonitrile (2:1; 0-15%, 33  
minutes) at a flow rate of 0.5 mL/min. Fractions were collected,  
lyophilized and later reconstituted with 100 mM NaCl, 20 mM Tris-  
HCl, pH 7.4. Inhibitory binding activity was quantitatively recovered.  
30 This material elutes at ca. 10% organic solvent and appears chromato-  
graphically pure. Samples were denatured in the presence of SDS and  
applied to precast 16% Tricine gels (NOVEX). The SDS-PAGE  
analysis of the active material using silver staining revealed a single  
component with an apparent molecular mass of ca. 4 kDa.

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### EXAMPLE 2

### Amino Acid Sequence Determination of MgTX

Purified MgTX, obtained above, was reduced, subjected to alkylation with iodoacetic acid and repurified by reverse-phase chromatography as previously described for ChTX in Gimenez-Gallego *et al. Proc. Natl. Acad. Sci. U.S.A.* 85, 3329, 1988. Briefly, purified MgTX was reduced in a medium consisting of 6M guanidine-HCl, 0.1% EDTA Na<sub>2</sub>, 0.1 M Tris-HCl, pH 9.5, 20 mM DTT by incubation at 50°C for two hours in the dark. Alkylation was then carried out in a medium consisting of 6M guanidine-HCl, 0.1% EDTA Na<sub>2</sub>, 0.7 M Tris-HCl, pH 7.8, 120 mM iodoacetic acid for 40 minutes at room temperature. The reaction mixture was applied to a C18 reversed phase column as employed for the purification of MgTX. Elution took place by applying a linear gradient of isopropyl alcohol/acetonitrile (2:1, 0-30%, 30 minutes). Approximately 0.5 nmoles of reduced-alkylated toxin was loaded onto a PORTON peptide support filter and Edman degradation was performed using a PORTON 2090 microsequencer. Phenylthiohydantoin derivatives were analyzed using an on-line detection system. Typical repetitive yields from at least six sequencing runs were 94%.

The primary amino acid sequence of MgTX is shown below:

(SEQ ID NO:1)

25 (SEQ ID NO.1)  
Thr-Ile-Ile-Asn-Val-Lys-Cys-Thr-Ser-Pro-Lys-Gln-Cys-Leu-Pro-Pro-  
5 10 15  
Cys-Lys-Ala-Gln-Phe-Gly-Gln-Ser-Ala-Gly-Ala-Lys-Cys-Met-Asn-Gly-  
30 20 25 30  
Lys-Cys-Lys-Cys-Tyr-Pro-His  
35

Because of the presence of several Proline residues, link steps were used at those positions to increase the efficiency of the

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cleavage reaction. From these data obtained in repetitive runs, 38 amino acid residues were well-defined. However, from our experience sequencing other peptides, we noted that the last residue of a peptide is frequently lost from the filter support and, in most cases, cannot be identified by this technique. Therefore, the molecular mass of MgTX was determined by electrospray mass spectroscopy. Mass spectral analysis of MgTX showed that the predominant component present in the preparation has a mass of 4178.0 Da. Since the combined mass of the 38 amino acid residues defined by sequence analysis only accounts for 4042.6 Da, we concluded that the carboxyl terminus of MgTX had not been identified. The difference in mass, 135.4, suggested histidine as the most likely candidate for the carboxyl terminal residue.

To resolve this issue, a sample of purified MgTX was subjected to amino acid hydrolysis. A sample containing 1 nmol of purified MgTX was subjected to acid hydrolysis, derivatized and phenylthiocarbamyl amino acid derivatives identified by reversed-phase chromatography using a PICO-TAG (WATERS) system. The absorbance spectrum of purified MgTX was digitized in a BECKMAN DU 7400 UV/Vis spectrophotometer. Protein extinction coefficients were calculated by determining the amino acid composition of an aliquot of MgTX and then correlating protein content with the recorded absorbance at 280, 235, and 215 nm, respectively. The result of this experiment is shown in Table I below.

25

Table I  
Amino Acid Analysis of Margatoxin

	Residues/mol Toxin	
	<u>Observed</u>	<u>Integer</u>
Aspartic acid or asparagine	1.80	2
Glutamic acid or glutamine	3.00	3
Serine	2.05	2
Glycine	3.15	3
Histidine	0.98	1
Arginine	NDa	
Threonine	1.97	2
Alanine	3.01	3
Proline	4.05	4

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	Tyrosine	1.02	1
	Valine	1.02	1
	Methionine	0.93	1
	Cysteine	5.92	6
5	Isoleucine	1.64	2
	Leucine	1.00	1
	Phenylalanine	1.04	1
	Lysine	5.94	6

NDA, not detected

10

The amino acid composition of MgTX corresponded well with that expected from the amino acid sequence determination, and, in addition, the presence of a single His residue was detected. These data defined MgTX as a 39 amino acid peptide with a carboxyl terminal histidine. The extinction coefficients determined for MgTX were: 12.82 cm<sup>-1</sup> x (mg/ml)<sup>-1</sup> at 215 nm, 2.59 cm<sup>-1</sup> x (mg/ml)<sup>-1</sup> at 235 nm, and 0.27 cm<sup>-1</sup> x (mg/ml)<sup>-1</sup> at 280 nm.

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### EXAMPLE 3

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Competition binding of MgTX to K<sub>v</sub>1.3 in rat brain synaptic plasma membrane vesicles, and to Maxi K channels in bovine aortic smooth muscle sarcolemmal membranes

The potency of MgTX as an inhibitor of [125I]ChTX binding to K<sub>v</sub>1.3 was evaluated. The binding of [125I]ChTX to either rat brain synaptic plasma membrane vesicles (K<sub>v</sub>1.3 channel; Vazquez *et al.*, *J. Biol. Chem.* **265**, 15564, 1990) or bovine aortic smooth muscle sarcolemmal membranes (Maxi-K channel; Vazquez *et al.*, *J. Biol. Chem.* **264**, 20402, 1989) was determined as described. Briefly, membrane vesicles were incubated with [125I]ChTX in the presence or absence of other added agents, until equilibrium was achieved. Separation of bound from free ligand was accomplished by filtration through GF/C glass fiber filters (WHATMAN) that had been presoaked in 0.5% polyethylene-imine. Nonspecific binding was determined in the presence of 10 nM unlabeled ChTX. Data from saturation experiments

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- 20 -

were analyzed in a Scatchard representation to determine  $K_d$  and  $B_{max}$  values. Triplicate samples were obtained for each experimental point and the data were averaged. The standard deviation of the mean was typically less than 2%. MgTX inhibited [ $^{125}$ I]ChTX binding to brain  
5 membranes in a concentration-dependent fashion with a Hill coefficient of 1.7 and an  $IC_{50}$  of 36 pM, under the low ionic strength conditions (50mM NaCl, 20 mM Tris-HCl, pH 7.4) used in this assay. As an indication of selectivity, MgTX had no effect on [ $^{125}$ I]ChTX binding to  
10 the Maxi-K channel in smooth muscle membranes at concentrations up to 10 nM. Saturation binding experiments employing increasing concentrations of [ $^{125}$ I]ChTX in the presence of fixed amounts of MgTX indicated that the inhibition of ChTX binding was due to a mixed effect on ligand affinity and receptor density. Under control conditions,  
15 [ $^{125}$ I]ChTX was bound to a single class of sites with a  $K_d$  of 31 pM and a maximum density of 0.43 pmol/mg protein. In the presence of 25 pM MgTX, the  $K_d$  for [ $^{125}$ I]ChTX increased to 52 pM, while the receptor density diminished to 0.34 pmol/mg protein. Thus, it appears that  
20 MgTX does not behave as a strictly competitive inhibitor of ChTX binding to  $K_v1.3$  in rat brain.

#### EXAMPLE 4

##### Competition Binding of MgTX to $K_v1.3$ of Human T Lymphocytes

In addition to being tested for competition for [ $^{125}$ I]ChTX  
25 binding to rat brain and bovine aortic smooth muscle, MgTX was evaluated for its effects on [ $^{125}$ I]ChTX binding to human T lymphocytes and to plasma membranes prepared from Jurkat cells, a human T-cell leukemia line. MgTX competition for [ $^{125}$ I]ChTX binding to human T cells was performed as described by Lin, *et al.*, *J. Exp. Med.* 177, 637-645, 1993. Binding experiments using monocyte-depleted, E-rosetted human T cells were carried out in isotonic sucrose  
30 with 5 mM KCl, 5 mM NaCl, 6 mM glucose and 10 mM HEPES, pH 8.0 until equilibrium was achieved. The procedure for binding to plasma membranes from Jurkat Cells is similar except that the binding solution was 3 mM KCl, 7 mM NaCl, 10 mM HEPES, pH 8.0. Free ligand was

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removed from bound by filtration through Whatman GF/C glass fiber filters (pretreated with 0.5% polyethylenimine). [ $^{125}$ I]ChTX binding was completely inhibited by MgTX with IC<sub>50</sub>'s of 1.4 and 2.7 pM for human T cells and plasma membranes from Jurkat cells, respectively. Hill coefficients were 2 in both cases.

### EXAMPLE 5

#### Expression of Recombinant MgTX

In order to verify that the amino acid sequence determined for MgTX corresponds to the active component of the preparation, as well as to obtain significant quantities of the peptide, recombinant MgTX (rMgTX) was expressed in *E. coli* as part of a fusion protein with the T7 gene 9 product. After partial purification of the fusion protein and cleavage with factor X<sub>1</sub> protease, rMgTX was purified to homogeneity by ion-exchange and reversed-phase chromatography. Amino acid analysis and sequence determination indicated that rMgTX is identical to native peptide. Importantly, rMgTX displayed the same potency and selectivity as an inhibitor of K<sub>v</sub>1.3 (see Example 8 below). In addition, rMgTX displayed the same potency as native MgTX for inhibition of [ $^{125}$ I]ChTX binding to brain membranes, and did not affect ChTX binding to the Maxi-K channel. These results indicate that the active species of the purified preparation corresponds to MgTX, and not to some other minor peptidyl component. Moreover, the carboxyl terminus of MgTX does not require amidation for full biological activity of the peptide.

### EXAMPLE 6

#### Construction of the Margatoxin Plasmid

The MgTX gene was constructed using two synthetic oligonucleotides that were synthesized using an ABI model 391 DNA synthesizer (Applied Biosystems). The two synthetic oligonucleotides are shown below, except for the 5 dCTP bases that were added to the 5'

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end of each to facilitate cutting with restriction enzymes. (SEQ ID NO:3)

5' GTCGACGGAT CCATCGAAGG TCGTACCATC ATCAACGTTA  
 5 AATGCACCTC TCCGAAACAG TGCCTGCCGC CGTGCAAAGC TCAG 3'  
 (SEQ ID NO: 4)  
 5' GGCACGTTTC GAGTCAAGCC AGTCAGACGA CCACGATTTA  
 10 CGTACTTGCC ATTTACGTTT ACGATGGGCG TGACTATCCT TCGAA3'

The two sequences above were combined to produce the gene having the coding strand shown below: (SEQ. ID NO:5)

5' GTCGACGGAT CCATCGAAGG TCGTACCATC ATCAACGTTA  
 15 AATGCACCTC TCCGAAACAG TGCCTGCCGC CGTGCAAAGC  
 TCAGTTCGGT CAGTCTGCTG GTGCTAAATG CATGAACGGT  
 AAATGCAAAT GCTACCCGCA CTGATAGGAA GCTT 3'

20 Codon usage was optimized for high level expression in E. coli. The oligonucleotides were purified using NENSORB columns (DUPONT), annealed, filled in with dNTPs using SEQUENASE (U.S. BIOCHEMICAL), and digested with restriction enzymes using standard techniques. The resulting fragment was gel purified using NUSIEVE  
 25 (FMC BIOPRODUCTS) and ligated into plasmid pCSP105, a variant of pSR9 using standard techniques as described in Ausubel et al., eds. (1989) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York. The resulting construct, encoding a fusion protein of T7 gene 9 and MgTX separated by a factor X<sub>a</sub> cleavage site was  
 30 verified using dideoxy sequencing.

## EXAMPLE 7

### Expression and Purification of Recombinant Margatoxin



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E. coli BL21(DE3) harboring p6MgTX were cultured and induced with IPTG. Purification of the fusion protein was done essentially as described in Park, et al. *Proc. Natl. Acad. Sci. U. S. A.* 88, 2046, 1991. Briefly, induced cells were pelleted at 4,000xg for 10 minutes, washed once in 50 mL of phosphate buffered saline and stored overnight at -80°C. Cells were thawed on ice and resuspended in 20 mL of 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1mM Na<sub>2</sub>EDTA, 1 mM DTT and protease inhibitors (100 µM PMSF, 1 µg/mL pepstatin, 1 µg/mL leupeptin), and incubated with 0.5 mg/mL lysozyme. Cells were disrupted by sonication and the lysate was centrifuged at 27,000xg for 15 min. After removal of nucleic acids with streptomycin sulphate, the lysate was loaded onto a DEAE-SEPHAROSE column equilibrated in 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM DTT. After washing the column, the fusion protein was eluted with 350 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM DTT. Fractions containing the fusion protein were identified by Coomassie staining of SDS-PAGE gels, combined, and dialyzed overnight against 20 mM Tris-HCl, 100 mM NaCl, 0.5 mM β-mercaptoethanol, pH 8.3. CaCl<sub>2</sub> was then added to a final concentration of 3 mM, and 1 µg Factor X<sub>a</sub> was added per 200 µg of fusion protein. The digestion mixture was incubated at room temperature for 18 hours. The recombinant MgTX (rMgTX) was purified from the cleavage mixture using MonoS FPLC and C18 reversed phase HPLC columns as described in EXAMPLE 1 for native MgTX. The yield was 3-4 mg of purified rMgTX per liter of culture. The composition of the purified material was verified by Edman degradation and amino acid hydrolysis.

### EXAMPLE 8

#### Electrophysiological measurements

Ionic currents were recorded from human peripheral T-lymphocytes using the whole cell patch clamp technique described in Cahalan, M. and Neher, E., *Meth. Enzymol.* 207, 3, 1992. Purified human T cells were obtained as described in Leonard et al., *Proc. Natl. Acad. Sci. U.S.A.*, 89, 10094, 1992, and recordings were carried out in

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a bath solution consisting of 125 mM NaCl, 2.5 mM KCl, 1.0 mM CaCl<sub>2</sub>, 2.0 mM MgCl<sub>2</sub>, 10 mM HEPES (potassium salt), 10 mM glucose, 5 mM Na-pyruvate, and 0.05% BSA. The pH was adjusted to 7.2, and experiments were performed at room temperature (19-22°C).  
5 The intracellular (pipette) solution contained 140 mM K-glutamate, 1 mM MgCl<sub>2</sub>, 10 mM HEPES (potassium salt), and 0.1 mM EGTA. The pH was adjusted to 7.2. Patch pipettes were fabricated from DAGAN (Minneapolis, MN) LG-16 glass and had resistances of 2 to 8 MΩ. Currents were measured using a DAGAN model 3900 patch clamp.  
10 Delivery of command voltages, data acquisition, and post-acquisition analysis were aided by a microcomputer running the Pclamp program suite (AXON INSTRUMENTS, Foster City, CA).

Ionic currents were also recorded from *Xenopus* oocytes that had been injected with RNA transcribed *in vitro* from one or  
15 another of the following K<sup>+</sup> channel cDNA clones. K<sub>v</sub>1 (rat K<sub>v</sub>1.5), rat or human K<sub>v</sub>3 (rat K<sub>v</sub>1.3; human K<sub>v</sub>1.3), K<sub>v</sub>4 (rat K<sub>v</sub>3.1), rat ISK, K<sub>v</sub>2 (rat K<sub>v</sub>1.6) or *Drosophila* Shaker-H4. The names of the clones are listed as originally published, with the appropriate parenthetical designation according to the unified gene nomenclature proposed by  
20 Chandy. Shaker-H4 is a splice variant from the original *Drosophila* Shaker K<sup>+</sup> channel gene. K<sub>v</sub>1.3 is the ChTX-sensitive delayed rectifier current found in rat and human T lymphocytes (data from rat and human clones were indistinguishable and were combined) and it is a member of the Shaker family. K<sub>v</sub>1.5 is a channel cloned from rat  
25 (Swanson *et al.*, *Neuron*, 4, 929, 1990) and is another member of the Shaker family. The K<sub>v</sub>1.5 transcript has been detected in pancreatic β cells as described in Philipson, *et al.*, *Proc. Natl. Acad. Sci. U. S. A.* 88, 53, 1991. K<sub>v</sub>1.6 is a clone from rat (Swanson *et al.*, *Neuron*, 4, 929, 1990) that shows 86% homology with K<sub>v</sub>1.3. Unlike K<sub>v</sub>1.3, this  
30 channel is very insensitive to ChTX, with an IC<sub>50</sub> > 1 μM. It is another member of the Shaker family. K<sub>v</sub>3.1 is a channel cloned from rat (Luneau *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 88, 3932, 1991) that appears to encode a ChTX-insensitive delayed rectifier found in thymic cells (the *I*-current) (Chandy *et al.*, *Biophys. J.*, 61, A257, 1992)). It is

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a member of the Shaw subfamily. ISK is a slowly-activating K<sup>+</sup> channel cloned from smooth muscle and heart (Folander *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 2975, 1990). This channel appears to underlie the I<sub>Ks</sub> delayed rectifier in heart muscle (Sanguinetti and Jurkiewicz, *Am. J. Physiol.*, **260**, J393, 1991; Sanguinetti and Jurkiewicz, *J. Gen. Physiol.*, **96**, 195, 1990). It is structurally unrelated to the other voltage-activated K<sup>+</sup> channel clones. The ISK transcript has been detected in lymphocytes (Attali *et al.*, *J. Biol. Chem.*, **267**, 8650, 1992), but no evidence of the I<sub>Ks</sub> current has been found in those cells.

Methods for oocyte isolation and micro-injection were previously described in Goldin, *Meth. Cell Biol.*, **36**, 487, 1991, and Swanson and Folander, *Meth. Enzymol.*, **207**, 310, 1992. Currents expressed in oocytes were measured using a DAGAN model CA-1 two-electrode voltage clamp. Delivery of command voltages, data acquisition and post-acquisition analysis were aided by a microcomputer running the Pclamp program suite. The oocyte bathing solution consisted of 96 mM NaCl, 2.5 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 10 mM HEPES, and 0.05% BSA. The pH was 7.2. All recordings were obtained at room temperature (19-22°C). The intracellular microelectrodes were fabricated from DAGAN LE-16 glass in a two-stage pull to a final resistance of 0.6 to 2 MΩ and filled with 3 M KCl. For both lymphocyte and oocyte experiments, peptides were diluted into the extracellular solution from 20 μM aqueous stock containing 100 mM NaCl. The presence of 0.05% BSA in the bath solution inhibited non-specific loss of dilute peptide toxins in the perfusion tubing.

Both native and recombinant MgTX were tested for their ability to block voltage gated K<sup>+</sup> channels. The two preparations gave identical results on K<sub>v</sub>1.3. There was insufficient native toxin to carry out selectivity studies, so those were conducted using the recombinant material exclusively. Complete inhibition of the voltage activated K<sup>+</sup> channel from human lymphocytes occurs at 1 nM, and recovery from block, following removal of MgTX from the bath, proceeds slowly, with a t<sub>1/2</sub> of >20 minutes. The apparent IC<sub>50</sub> for inhibition of the

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lymphocyte  $K_v1.3$  current is between 20 to 100 pM. This is five to ten-fold more potent than ChTX on the same preparation. As reported previously, MgTX has no effect on three different ChTX-sensitive,  $Ca^{2+}$ -activated  $K^+$  channels of lymphocytes, even at 100 nM (Leonard et al., *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 10094, 1992).

Of the six cloned channels tested, three ( $K_v1.5$ ,  $K_v3.1$ , and ISK) were completely insensitive to 200 nM MgTX. The most sensitive channel is  $K_v1.3$ , with an  $IC_{50}$  of ~30 pM. The next most sensitive channel,  $K_v1.6$ , was half blocked at 5 nM, while Shaker-H4 displayed an  $IC_{50}$  of 100 nM.

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EXAMPLE 9Iodination of Margatoxin

Two reaction mixtures were prepared in individual microcentrifuge tubes (1.5 mL), each containing the following (in a total volume of 0.065 mL): ENZYMOBEADS<sup>®</sup> (the contents of a single reaction vial, rehydrated according to the manufacturer's instructions), 200 mM sodium phosphate, pH 7.0, and 30.8  $\mu$ M recombinant MgTX. The reaction mixtures were allowed to equilibrate at room temperature for approximately 15 minutes. Then, Na<sup>125</sup>I (2 mCi; approx. 6  $\mu$ L) was added to each tube, followed by a solution of  $\beta$ -D-glucose (5  $\mu$ L of a 50 mg/mL solution in water). The contents of the tubes were mixed by gentle tapping. Incubation was for 20 min at room temperature. The tubes were then opened and allowed to vent for 1 min. The reaction mixtures were filtered on a COSTAR microcentrifuge filter (0.22  $\mu$ m). Each tube was washed with sodium phosphate, pH 7.0 (0.15 mL) and the washes centrifuged through the same filter as the reaction mixtures. Finally, the filter was washed with buffer (0.075 mL). The reaction supernatants and washes were combined and loaded onto a VYDAC C18 Protein & Peptide column (0.46 x 25 cm) that had been equilibrated at room temperature with 5% solvent B(2:1 isopropanol:acetonitrile, 4 mM trifluoroacetic acid)/95% solvent A (10 mM trifluoroacetic acid). After unreacted sodium iodide had been eluted by washing the column with the equilibration solvent for 25 min at 0.8 mL/min, the unreacted MgTX and its iodinated products were separated by a linear gradient of (9-15)% solvent B over a period of 30 min. Two protein peaks containing radioactivity were detected. The second, smaller peak (Diiodinated margatoxin) was used in the binding assay.

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EXAMPLE 10Competition binding of MgTX to K<sub>v</sub>1.3 of human T lymphocytes

5        Diiodinated margatoxin, [<sup>125</sup>I]2MgTX, was used to develop binding assays for K<sub>v</sub>1.3 in human T lymphocytes and in plasma membranes prepared from Jurkat cells. [<sup>125</sup>I]2MgTX was incubated either with intact monocyte-depleted, e-rosetted human peripheral blood T lymphocytes or with Jurkat plasma membranes in a solution containing 4.6 mM KCl, 106 mM NaCl, 20 mM HEPES, pH 7.4 until equilibrium was reached. Experiments in cells were performed at ambient temperature (from 21 to 25°C) while the plasma membranes were incubated at 37°C. Free [<sup>125</sup>I]2MgTX was removed from bound by passing the reaction mixture through WHATMAN GF/C glass fiber filters which had been presoaked with 0.5% polyethylenimine. The radioactivity trapped on these filters was determined using gamma-counting techniques. When this procedure was used for [<sup>125</sup>I]2MgTX binding to Jurkat plasma membranes, the equilibrium constant (K<sub>d</sub>) was 8 pM and maximum binding (B<sub>max</sub>) was 64 fmol per mg protein. 15        [<sup>125</sup>I]2MgTX binding to human T cells exhibited a K<sub>d</sub> of 15 pM and a B<sub>max</sub> of 800 sites/cell. 20

EXAMPLE 1125    Formulation of Margatoxin

An injectable formulation is prepared as follows:

Margatoxin	1 mg
Polysorbate 80	10 µg
30 Sodium chloride	10 mg
Distilled water for injection	q.s.
per vial of 1 mL	

The formulation is filtered to remove organisms and dispensed into a 2 mL glass vial.

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While the foregoing specification teaches the principles of the present invention, with examples provided for the purpose of illustration, it will be understood that the practice of the invention encompasses all of the casual variations, adaptations, modifications, deletions or additions of procedures and protocols described herein, as come within the scope of the following claims and its equivalents.

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- 30 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: GARCIA, MARIA L.  
KOO, GLORIA C.  
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STEVENS, SCOTT P.  
WILLIAMSON, JOANNE M.
- (ii) TITLE OF INVENTION: SCORPION PEPTIDE WITH IMMUNOSUPPRESSANT  
ACTIVITY
- (iii) NUMBER OF SEQUENCES: 5
- (iv) CORRESPONDENCE ADDRESS:
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  - (D) STATE: NEW JERSEY
  - (E) COUNTRY: USA
  - (F) ZIP: 07065-0907
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: US
  - (B) FILING DATE: 22-JUL-1993
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
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  - (B) REGISTRATION NUMBER: 36356
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  - (C) TELEX: 138825

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 39 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear



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(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: MARGATOXIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Thr Ile Ile Asn Val Lys Cys Thr Ser Pro Lys Gln Cys Leu Pro Pro  
1 5 10 15

Cys Lys Ala Gln Phe Gly Gln Ser Ala Gly Ala Lys Cys Met Asn Gly  
20 25 30

Lys Cys Lys Cys Tyr Pro His  
35

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 127 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ACCATCATCA ACGTTAAATG CACCTCTCCG AAACAGTGCC TGCCGCCGTG CAAAGCTCAG 60  
TTCGGTCAGT CTGCTGGTGC TAAATGCTAA ATGCATGAAC GGTAAATGCA AATGCTACCC 120  
GCACTGA 127

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 84 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTCGACGGAT CCATCGAAGG TCGTACCATC ATCAACGTTA AATGCACCTC TCCGAAACAG 60  
TGCCTGCCGC CGTGCAAAGC TCAG 84

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 85 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGCAGCTTTC GAGTCAAGCC AGTCAGACGA CCACGATTTA CGTACTTGCC ATTTACGTTT 60  
ACGATGGGCG TGA CTATCCT TCGAA 85

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 154 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTCGACGGAT CCATCGAAGG TCGTACCATC ATCAACGTTA AATGCACCTC TCCGAAACAG 60  
TGCCTGCCGC CGTGCAAAGC TCAGTTCGGT CAGTCTGCTG GTGCTAAATG CATGAACGGT 120  
AAATGCAAAT GCTACCCGCA CTGATAGGAA GCTT 154

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WHAT IS CLAIMED IS:

1. A purified peptide comprising the sequence (SEQ ID  
5 NO:1)  
Thr-Ile-Ile-Asn-Val-Lys-Cys-Thr-Ser-Pro-Lys-Gln-Cys-Leu-Pro-Pro-  
5 10 15  
Cys-Lys-Ala-Gln-Phe-Gly-Gln-Ser-Ala-Gly-Ala-Lys-Cys-Met-Asn-Gly-  
10 20 25 30  
Lys-Cys-Lys-Cys-Tyr-Pro-His.  
35
2. The purified peptide of Claim 1 wherein the peptide  
15 is produced by recombinant techniques.
3. The purified peptide of Claim 1 wherein the peptide  
is produced by recombinant techniques using E. coli cells.
- 20 4. A composition for assessing the contribution of  
K<sub>v</sub>1.3 voltage dependent potassium channels in neurons, blood cells,  
osteoclasts and T-cells comprising the purified peptide of Claim 1.
- 25 5. A composition for assessing the contribution of  
K<sub>v</sub>1.3 voltage dependent potassium channels in T-cells comprising the  
purified peptide of Claim 1.
- 30 6. A pharmaceutical composition comprising an  
immunosuppressive amount of the peptide of Claim 1 and a  
pharmaceutically acceptable carrier.
7. An injectable pharmaceutical composition comprising  
0.001% to 10% (w/v) of the peptide of Claim 1 and a pharmaceutically  
acceptable carrier.

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8. The injectable pharmaceutical composition of Claim 7 wherein the carrier comprises aqueous sodium chloride.

5 9. A method of suppressing the immune system in a mammalian subject in need of such treatment comprising the administration of a therapeutically effective amount of the peptide of Claim 1 to the subject.

10 10. A method for the treatment of immunoregulatory disorders or diseases comprising the administration to a mammalian species in need of such treatment of an effective amount of the peptide of Claim 1.

15 11. A method for the treatment of resistance to transplantation comprising the administration to a mammalian species in need of such treatment of an effective amount to the peptide of Claim 1.

20 12. A DNA encoding the peptide of Claim 1 which is free from other coding DNA sequences.

13. The DNA according to Claim 12 comprising the sequence: (SEQ ID NO:2)

25 5' ACCATCATCA ACGTTAAATG CACCTCTCCG AACAGTGCC  
TGCCGCCGTG CAAAGCTCAG TTCGGTCAGT CTGCTGGTGC  
TAAATGCATG AACGGTAAAT GCAAATGCTA CCCGCACTGA 3'

or a degenerate variant thereof.

30 14. A synthetic oligonucleotide useful to make the gene encoding the peptide of Claim 1 comprising the sequence selected from (SEQ ID NO:3).

5' GTCGACGGAT CCATCGAAGG TCGTACCATC ATCAACGTTA

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AATGCACCTC TCCGAAACAG TGCCTGCCGC CGTGCAAAGC TCAG 3'  
and  
(SEQ ID NO: 4)

5 5'GGCACGTTTC GAGTCAAGCC AGTCAGACGA CCACGATTTA  
CGTACTTGCC ATTTACGTTT ACGATGGGCG TGA CTATCCT TCGAA3'

or a degenerate variant thereof.

10 15. A synthetic oligonucleotide coding for the peptide of  
Claim 1, comprising the sequence: (SEQ ID NO:5)

5' GTCGACGGAT CCATCGAAGG TCGTACCATC ATCAACGTTA  
AATGCACCTC TCCGAAACAG TGCCTGCCGC CGTGCAAAGC  
15 TCAGTTCGGT CAGTCTGCTG GTGCTAAATG CATGAACGGT  
AAATGCAAAT GCTACCCGCA CTGATAGGAA GCTT 3'

or a degenerate variant thereof.

20 16. An expression construct which comprises: a cell  
vector, and the base sequence encoding Margatoxin peptide according to  
Claim 12.

25 17. An expression construct which comprises: an  
inducible promoter of transcription, a selectable marker for antibiotic  
resistance and the base sequence encoding Margatoxin peptide according  
to Claim 12.

30 18. The expression construct of Claim 17 wherein the  
selectable marker for antibiotic resistance is plasmid PCSP105.

19. The expression construct according to Claim 17  
wherein the base sequence encoding MgTX peptide comprises the  
sequence: (SEQ ID NO:2)

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5' ACCATCATCA ACGTTAAATG CACCTCTCCG AAACAGTGCC  
 TGCCGCCGTG CAAAGCTCAG TTCGGTCAGT CTGCTGGTGC  
 TAAATGCATG AACGGTAAAT GCAAATGCTA CCCGCACTGA 3'

or a degenerate variant thereof.

20. The expression construct according to Claim 18  
 which comprises the sequence: (SEQ ID NO:5)

5' GTCGACGGAT CCATCGAAGG TCGTACCATC ATCAACGTTA  
 AATGCACCTC TCCGAAACAG TGCCTGCCGC CGTGCAAAGC  
 TCAGTTCGGT CAGTCTGCTG GTGCTAAATG CATGAACGGT  
 AAATGCAAAT GCTACCCGCA CTGATAGGAA GCTT 3'

or a degenerate variant thereof.

21. A cell line competent for transfection with foreign  
 DNA which is transfected with an expression construct according to  
 Claim 16.

22. An E. coli cell line which is transfected with an  
 expression construct according to Claim 16.

23. A BL21(DE3) E. coli cell line which is transfected  
 with an expression construct according to Claim 16.

24. A radiolabelled peptide according to Claim 1.

25. A peptide having the amino acid composition:

	<u>Residues/mol Toxin</u>
Aspartic acid or asparagine	2
Glutamic acid or glutamine	3
Serine	2

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	Glycine	3
	Histidine	1
	Threonine	2
	Alanine	3
5	Proline	4
	Tyrosine	1
	Valine	1
	Methionine	1
	Cysteine	6
	Isoleucine	2
10	Leucine	1
	Phenylalanine	1
	Lysine	6.

26. A biochemical assay to determine whether a molecule inhibits the binding of the peptide of Claim 1 to K<sub>v</sub>1.3 channels in cells expressing K<sub>v</sub>1.3 channels or to plasma membrane vesicles derived from the cells comprising:

- (a) incubating radiolabelled MgTX with lymphocytes or plasma membranes in buffer containing the molecule;
- (b) filtering the mixture above to remove free radiolabelled MgTX from bound;
- (c) measuring the radioactivity on the filter; and
- (d) comparing the result with the result obtained when MgTX is incubated in the presence of buffer alone.

1/1

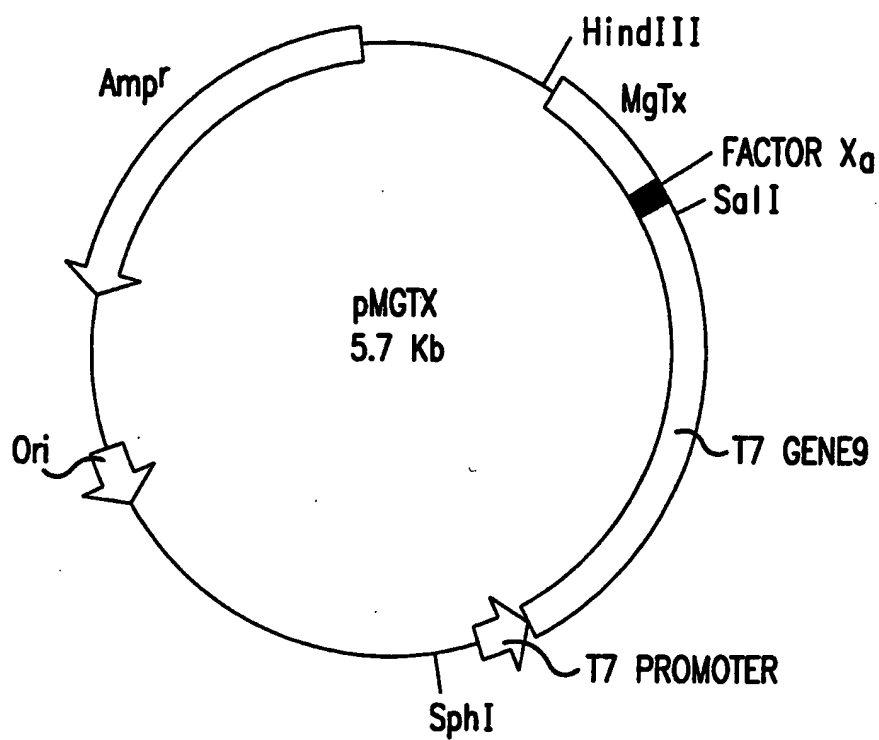


FIG.1



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/08063

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>												
IPC(5) : Please See Extra Sheet.												
US CL : 424/1.69; 435/7.1, 69.1, 252.33, 320.1; 514/12; 530/324; 536/23.5												
According to International Patent Classification (IPC) or to both national classification and IPC												
<b>B. FIELDS SEARCHED</b>												
Minimum documentation searched (classification system followed by classification symbols)												
U.S. : 424/1.69; 435/7.1, 69.1, 252.33, 320.1; 514/12; 530/324; 536/23.5												
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched												
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)												
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>												
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.										
X ----- Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Volume 89, issued November 1992, Leonard et al, "Selective blockers of voltage-gated K <sup>+</sup> channels depolarize human T lymphocytes: Mechanism of the antiproliferative effect of charybdotoxin", pages 10094-10098, see page 10094, columns 1-2, and paragraph bridging pages 10096-10097.	1-5, 25, 26 ----- 6-8, 24										
X ----- Y	BIOPHYSICAL JOURNAL, Volume 59, issued 1991, Novick et al, "Purification And Characterization Of Two Novel Peptidyl Toxins Directed Against K <sup>+</sup> Channels From Venom Of New World Scorpions", page 78, abstract M-Pos124, see entire abstract.	1-5, 25 ----- 6-8, 24										
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.												
* Special categories of cited documents: <table border="0"> <tr> <td>*A* document defining the general state of the art which is not considered to be of particular relevance</td> <td>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>*E* earlier document published on or after the international filing date</td> <td>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>*L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>*O* document referring to an oral disclosure, use, exhibition or other means</td> <td>*Z* document member of the same patent family</td> </tr> <tr> <td>*P* document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </table>			*A* document defining the general state of the art which is not considered to be of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	*E* earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	*L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	*O* document referring to an oral disclosure, use, exhibition or other means	*Z* document member of the same patent family	*P* document published prior to the international filing date but later than the priority date claimed	
*A* document defining the general state of the art which is not considered to be of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention											
*E* earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone											
*L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art											
*O* document referring to an oral disclosure, use, exhibition or other means	*Z* document member of the same patent family											
*P* document published prior to the international filing date but later than the priority date claimed												
Date of the actual completion of the international search		Date of mailing of the international search report										
24 AUGUST 1994		SEP 02 1994										
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231		Authorized officer										
Facsimile No. (703) 305-3230		STEPHEN WALSH <i>John Warden for</i>										
		Telephone No. (703) 308-0196										

# INTERNATIONAL SEARCH REPORT

I. International application No.  
PCT/US94/08063

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Volume 88, issued March 1991, Park et al, "Design, synthesis, and functional expression of a gene for charybdotoxin, a peptide blocker of K <sup>+</sup> channels", pages 2046-2050, see Materials and Methods, pages 2046-2047.	3, 4, 12-23
Y	US, A, 4,929,718 (POSSANI ET AL) 29 May 1990, see column 1, lines 26-58, and paragraph bridging columns 9-10.	6-11
A	US, A, 5,006,512 (OHNISHI) 09 April 1991, see abstract.	6-11

# INTERNATIONAL SEARCH REPORT

national application No.  
PCT/US94/08063

## A. CLASSIFICATION OF SUBJECT MATTER: IPC (5):

A61K 37/02, 43/00; C07K 7/10; C12N 1/21, 15/03, 15/12, 15/70; G01N 33/53